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Description and identification of two new diseases of guariroba palm (*Syagrus oleraceae*) in Brazil

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Abstract

Guariroba is a palm species native to central Brazil. Seedlings of guariroba with leaf spots of unknown aetiology were found in Patos de Minas, Minas Gerais, Brazil. The leaf spots were manifest as two different symptom types: the first lesion type consisted of necrotic spots with a rounded to elongate shape, with a light colour and dark edges; the second lesion type had a rounded shape, was dark brown in colour with a light brown edge. The objective of this study was to elucidate the aetiology of both diseases. The likely causal agents were isolated and Koch's postulate fulfilled. Subsequently, the ITS region of rDNA from both micro-organisms were amplified and sequenced. According to the morphological characteristics and molecular analyses, the fungal species were identified as *Pestalotiopsis adusta* (causing necrotic spots with a rounded to elongate shape, with a light colour and dark edges), and *Alternaria tenuissima* (causing lesions with a rounded shape, dark brown in colour with a light brown edge). Identification of the causal organisms of these two diseases will help guide management approaches that might be tested to reduce impact of the disease on Guariroba, including the use of fungicides and cultural approaches.

KEYWORDS

Alternaria tenuissima, guariroba, Koch's postulates, *Pestalotiopsis adusta*, *Syagrus oleracea*

1 | INTRODUCTION

Guariroba (*Syagrus oleracea* (Mart.) Becc.) is a native palm of the Midwest region of Brazil, belonging to the family Arecaceae, subfamily Cocosoideae (Pinto, Reis, Faleiro, Barbosa, & Nunes, 2009). Guariroba is cultivated for bitter palm heart, and is consumed mostly locally in the states of Goiás, Tocantins, Mato Grosso, Mato Groso du Sul and some parts of Minas Gerais and the Federal District (de Aguiar & de Almeida, 2000; Carneiro, Rolim, & Fernandes, 2003). Until recently, the culture of guariroba has been mostly extractive from naturally growing trees, and this has led to a decline of its native populations (de Aguiar & de Almeida, 2000). Consequently, cultivation of guariroba has increased, and commercial plantations of guariroba are becoming more widespread. This is particularly true in the state of Goias, where it was estimated 4,499 ha of guariroba were grown in 1999, mostly in mixed cropping systems (de Aguiar & de Almeida, 2000). That study reported that in the municipality of Aragoiania (in Goias), although only 0.3% of the area was cultivated to guariroba, it was the second most economically important crop in that region (accounting for 15.4% of the economy). These authors also found that over an 8 year period, the benefit to cost ratio of guariroba production was 7.12, substantially higher than the ratio for citrus (4.96), corn (1.14) or rice (0.81), the three most widely cultivated crops in the municipality. Furthermore, although guariroba is only a proportion of the total forest economy, it is estimated that plant extraction, forest regeneration and production in Brazil amounted to US$ 6.4 billion in 2014, (IBGE - Instituto Brasileiro de Geografia e Estatística, 2014).

In addition to the palm hearts, other parts of the palm can also be used (Lorenzi, Souza, Cerqueira, Ferreira, & Costa, 2004). The leaves are used for cattle feed, which have more than triple the

protein content and similar digestibility to those of *Brachiaria decumbens* (de Almeida, Bonnas, Jordão, & de Aguiar, 2000). The fruit and nuts can be consumed too, and the oil has a high commercial value (Dias, Laureano, & Ming, 2014). In view of the economic significance of guariroba, and the plant's suitability for cultivation (Melo, 2003), guariroba production has emerged as an excellent option for small farms, especially when the aim is to add value and diversify production, increase biodiversity and contribute to sustainable agriculture (de Aguiar & de Almeida, 2000; de Almeida et al., 2000; de Andrade et al., 2013; Dias et al., 2014; Lorenzi et al., 2004).

The health of cultivated plants is important to ensure yield and maximize economic success. Plant diseases can cause reduction in crop productivity, and to establish control strategies and avoid losses, a first step is to correctly diagnose the cause of diseases (Agrios, 2005). Few diseases afflict guariroba. Among those described on guariroba are anthracnose, caused by *Colletotrichum gloeosporioides* (Charchar, Anjos, & Akimoto, 2002), leaf spot, caused by *Cladosporium perangustum* (Oliveira et al., 2014) and a root disease caused by nematodes (*Pratylenchus brachyurus*) (Araújo Filho, Castro-Moretti, & Bonfim Junior, 2014). With the increase in cultivation of guariroba, disease issues are likely to become more widespread, as already noted in several new reports of pathogens infecting the crop (Araújo Filho et al., 2014; Oliveira et al., 2014). Indeed, with the increased production of guariroba it is necessary to characterize the factors that adversely affect production and to manage these issues appropriately to minimize their impact on the productivity of guariroba plantations. Diseases might impact the uniformity of plant growth, reducing yield and perhaps result in a later and more costly harvest time (Nascente, 2003). In addition, diseases may also affect the quality of palm heart harvested from guariroba.

Considering the increasing importance and commercialization of guariroba, characterizing diseases and their impact will be of immediate value in helping reduce any impact they have on this crop—only by knowing the causal organisms of disease can informed and efficacious approaches be developed to reduce their impact. Recent surveys (William M.C. Nunes, unpublished results) in seedling nurseries of guariroba in the state of Minas Gerais have found foliage showing a high prevalence of leaf spots of apparently unknown aetiology. The leaf spots were noted to occur at an incidence of 32.4%, with a range of severity on the foliage. The primary aim of this study was to characterize and describe the causal agents of these diseases in guariroba plants in Minas Gerais.

2 | MATERIAL AND METHODS

2.1 | Diseased hosts

Thirty eighteen-month-old seedlings of guariroba with symptoms of leaf spots were collected from each of two nurseries in the Patos de Minas region of Minas Gerais. Each nursery had approximately 30% of the plants showing symptoms of these diseases. The severity of disease was not measured directly, but ranged from a trace to approximately ≤20% (William M.C. Nunes, personal observation). The seedlings were transported to Maringa (Parana state) and placed in a greenhouse at the State University of Maringá (UEM, Maringa, Brazil) under natural light and temperature (24°C) conditions for further studies of the possible causes of the symptoms on the leaves. The studies were conducted at the Phytopathology Laboratory of the Agronomy Department of UEM.

2.2 | Symptomatology

Under close examination in the greenhouse, two distinct symptoms were observed on the foliage, possibly indicating two different causal agents. The study proceeded to characterize the two symptom types that were provisionally designated as "symptom type 1" and "symptom type 2." The two types of symptoms were assessed visually and observed under a dissecting microscope (×40). The symptomatic area on the leaf surface was scraped with a fine-tipped scalpel. This scraped material was deposited on a slide into a drop of water which was overlaid with a coverslip. Observations of any structures were made under an optical microscope (×40, using differential interference contrast microscopy, Zeiss Axioscope 2 PLUS, Oberkochen, Germany) to check for any structures that might be indicative of the causal agent.

2.3 | Isolation of the causal agents associated with guariroba leaf spots

The leaves collected were washed in sodium hypochlorite (0.2%) for 1 min, rinsed under running water and wiped dry with a paper towel and allowed to dry. The leaves were placed in a plastic box lined with moist paper towels at 25 ± 2°C for 24 hr with a 12-hr photoperiod. Subsequently, the symptoms were viewed under a microscope, and samples were taken from the leaf surface as described above, after viewing under a dissecting microscope (×40) as described above, samples were taken for direct isolation. Structures of the putative fungal pathogen present in the central area of the necrotic tissue were transferred with the aid of fine-pointed needle to Petri dishes containing potato dextrose agar (PDA) amended with 250 mg/L ampicillin. The Petri dishes were placed under fluorescent light with a 12-h photoperiod at a temperature of 25 ± 2°C, and were incubated for 5 days, when a mycelial colony had formed.

Plates on which a mycelial colony had become established without evidence of contamination (four cultures from symptom type 1, and five cultures from symptom type 2) were selected, and discs of agar containing only hyphal tips from the edge region of the colonies were removed. The discs were transferred to fresh Petri dishes containing PDA and maintained under the same conditions described above. The fresh colonies were used for morphological characterization, DNA extraction and production of inoculum for pathogenicity tests with re-isolation to fulfil Koch's postulate.

2.4 | Pathogenicity on leaf sections

In this study, pathogenicity tests were conducted with the four cultures from symptom type 1, and the five cultures from symptom type

2 using mycelium on agar discs placed on segments of detached leaves of guariroba.

2.4.1 | Production of inoculum

The isolations from the two symptom types described in the previous section were transferred to new Petri dishes containing PDA by taking plugs of agar containing mycelium and placing them singly in the centre of the new culture plates. These plates were incubated under the same conditions as previously described for 7 days to provide the inoculum for the experiment.

2.4.2 | Leaf material for inoculation

Healthy guariroba leaves were detached from 1-year-old seedlings. The leaves were washed and rinsed in tap water. The leaves were dried at room temperature, disinfected in sodium hypochlorite (0.2%) for 1 min, rinsed with sterile distilled water, wiped dry and cut into 8.0 cm long segments. The three segments from the middle section of each leaf were placed in a plastic acrylic box (Gerbox, Londrina, Brazil), lined with three sheets of absorbent paper (Germitest, Londrina, Brazil) moistened with sterile distilled water, which thus acted as a humid chamber.

2.4.3 | Inoculation

Wounds were made in the centre of the top surface of each leaf section using a set of six needles embedded in a cork stopper. The needles were arranged in a 10 mm diameter circle. A 5.0 mm diameter disc of PDA containing hyphae and spores taken from the edge of the 7-day-old colony was placed upside down on the wounded area so that the surface containing the fungal structures was in contact with the leaf surface. Three leaf sections were inoculated with each of the isolates. A control treatment consisted of leaves that were similarly injured and had an agar plug without fungus placed on the injured area. The leaf sections were placed in Gerbox containers as described above, and sealed with plastic film and placed under fluorescent lights with a 12-h photoperiod at 25 ± 2°C.

2.4.4 | Assessment

Leaf sections were assessed visually on a daily basis for symptom development over a period of 10 days after inoculation. The tenth day was the final evaluation. The presence/absence of symptoms was assessed, and lesion size was measured.

2.4.5 | Re-isolation of the fungus from the symptomatic leaf area

After the final assessment, re-isolation of the fungus from the symptomatic area on the leaf was performed using the same methodology described earlier. The resulting cultures were compared with those first isolated, and the morphology of the causal agent confirmed microscopically as described above, thereby fulfilling Koch's postulate.

2.5 | Pathogenicity test using seedlings of guariroba

In this experiment, two healthy seedlings were inoculated individually with spores from each of the four isolates obtained from lesions of symptom type 1 and from each of five isolates obtained from lesions of symptom type 2 (i.e., two seedlings were inoculated for each of the isolates = 8 seedlings inoculated with the four isolates of *P.* symptom type 1, and 10 seedlings inoculated for the five isolates of symptom type 2). Isolates were not mixed on plants.

2.5.1 | Production of inoculum

For isolates from symptom type 1, the inoculum was obtained growing the isolates on PDA as described, as it was suspected that this fungus was a *Pestalotiopsis* spp., but in this case, culture incubation time was 10 days (*Pestalotiopsis* spp. should produce spores on PDA (Kruschewsky, 2010). The isolates from symptom type 2 were suspected of being an *Alternaria* spp., and inoculum was obtained by transferring PDA discs containing mycelium to Petri dishes containing V8 culture medium (200 ml V8 juice, 16 g agar, 3.2 g $CaCO_3$ and 800 ml qs distilled water); Petri dishes were incubated under the same conditions described above.

2.5.2 | Inoculation

After 10 days of incubation, 10 ml of sterile distilled water was added to each Petri dish. With the aid of a Drigalski spatula, the surface of the medium was scraped to release spores. The resulting suspension was filtered through a double layer of muslin gauze to separate the spores from other culture-related material. The concentration of the spore suspension was adjusted to 1×10^6 spores/ml using a Hemocytometer (Neubauer chamber), followed by addition of 0.01% Tween 20. The guariroba seedlings used in the experiment had two to three healthy, fully expanded leaves, in the central area of which three equidistant injuries were made, following the same wounding procedure as described above for leaf sections. The spore suspension was applied by spraying the leaves until run-off with hand sprayer. A control was included and had leaves with injury but was sprayed with sterile distilled water. After inoculation, the plants were placed in a moist chamber for 72 hr before transfer to a plant growth chamber maintained at 25 ± 2° C, with fluorescent lighting and a 12-hr photoperiod.

2.5.3 | Evaluation

After 2 weeks incubation, the inoculated leaves on each plant were assessed qualitatively for disease development, as described above.

2.5.4 | Re-isolation of the fungus from the symptomatic leaf area

After the assessment of symptoms, the fungus was re-isolated as previously described, thereby fulfilling Koch's postulate.

2.6 | Identification of isolates obtained in pure culture

Using the pure cultures obtained from the isolations, PDA discs containing mycelium were transferred to fresh PDA in Petri dishes for isolates from symptom type 1 (suspected to be *Pestalodiopsis* spp.); for isolates from symptom type 2 (suspected to be *Alternaria* spp.), the discs were transferred to Petri dishes containing fresh V8 medium under the same conditions described above.

Cultures were allowed to grow until they were 3.0 cm in diameter, when two microcultures from each isolate were prepared on microscope slides. Square blocks (0.3 mm) of PDA (isolates from symptom type 1) and of V8 medium (for isolates of symptom type 2) were placed at the centre of a sterile microscope slide. With the aid of a fine point, hyphal filaments were transferred to each quadrant of the culture medium block, and were each covered with a sterile coverslip.

The microscope slide with each block of culture medium was supported in a Petri dish containing tissue paper moistened with sterile distilled water. The samples were incubated at 25 ± 2°C under fluorescent light with a 12-h photoperiod for 7 days. The coverslips were carefully removed and deposited on a slide with a drop of lactophenol for observation under an optical microscope (×40). Hyphae, conidiophores and spores present were characterized morphologically and measured with the aid of the image analysis software Motic Image Plus 2.0 (Richmond, British Columbia).

For each isolate, 40 spores were measured and compared with those structures described in the literature for the purpose of species identification. For the *Alternaria* spp., length, width and number of longitudinal and transverse septa were recorded. For the *Pestalotiopsis* spp., length, width, length between cells, number of appendices and length of appendices were recorded. A sample of each isolate was preserved using the Castellani method (Castellani, 1939), and placed in the fungal collection at the Phytopathology Laboratory of UEM.

2.7 | Confirmation of fungal species by sequencing

2.7.1 | Fungal culture and DNA extraction

Mycelial discs were removed from the edges of colonies of each isolate obtained from symptom type 1 and 2. The discs were transferred to Erlenmeyer flasks with 50 ml liquid medium (potato dextrose broth, PDB; 200 g potato extract, 18 g dextrose in 1,000 ml qs sterile distilled water). The cultures flasks were incubated at 25 ± 2°C for 7 days. The DNA was extracted following the methodology of White, Bruns, Lee, and Taylor (1990).

The mycelium from the liquid culture was filtered, macerated in liquid nitrogen with a mortar and pestle, and placed in sterile 1.5-ml microfuge tubes. Extraction buffer (100 mM Tris-HCl pH 8.0; 50 mM EDTA pH 8.0; 500 mM NaCl; 100 µl, 1% SDS) was added, and the samples were incubated in a water bath at 65°C for 30 min with agitation. Each sample immediately received 500 µl of 5 M potassium acetate and was incubated on ice for 30 min, stirring every 5 min. Samples were centrifuged for 10 min at 12,000-14,000 *g*. The supernatant was transferred to microfuge tubes, and chloroform and isoamyl alcohol (24:1, 500 µl) were added; tubes were again centrifuged for 10 min at 12,000-14,000 *g*. The supernatant was transferred to a fresh tube, and absolute ethanol (800 µl) was added and the tube allowed to stand for 15 min to precipitate the DNA.

The tube was again centrifuged for 10 min at 12,000-14,000 *g*, and the supernatant discarded, and 700 µl of 70% ethanol was added to the tube. Samples were suspended and centrifuged for a further 5 min and the supernatant again discarded. A further 400 µl of 100% ethanol was added and the tubes centrifuged for 5 min and the supernatant discarded. The tubes were inverted for 3 min to drain them with the caps open on a paper towel, to evaporate the ethanol. After, the dried pellets on the tubes were resuspended in 50 µl of TE + RNase (40 µl/ml) and incubated for 1 hr at 37°C. Quantification of DNA was performed and the concentration of DNA adjusted to 25 ng/µl by dilution with TE and the samples stored in the freezer (−20°C).

2.7.2 | Primers, PCR and sequencing

PCR was used to amplify regions of the internal transcribed spacer (ITS) of the ribosomal cistron for the two fungal species. A set of universal primers designed specifically for the ITS region ITS5 (3′ GGAAGTAAAAGTCGTAACAAGG 5′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) were used (White et al., 1990). The reaction mix (25 µl) contained 4.0 µl of fungal DNA (100 ng), 2.5 µl of T10x, 1.25 µl $MgCl_2$, 1.0 µl dNTPs (0,4 mM), 0.4 µl Taq polymerase (5U/µl), 1.0 µl of primers ITS4 and ITS5 (25 ng) and 13.85 µl ultrapure water. The mix was subject to 30 amplification cycles as follows: 94°C for 30 s, 65°C for 45 s and 72°C for 1 min. The reaction ended with a final extension period at 72°C for 5 min.

The PCR products were visualized under UV light using a 1% agarose gel stained with Ethidium Bromide (10 mg/ml) (Invitrogen, Carlsbad, CA, USA), and photodocumented. The PCR products were cleaned using an ExoSAP-IT PCR Clean-up Kit (GE Healthcare, Buckinghamshire, UK) and sent for sequencing (Institute of Biosciences, University of Sao Paulo, Sao Paulo, Brazil). The nucleotide sequences were analysed using the bioinformatics program "BioEdit", available on the European Bioinformatics Institute server (EBI, www.ebi.ac.uk). Finally the resulting sequences were BLAST searched against the database at GenBank for species confirmation.

3 | RESULTS

3.1 | Symptoms

Symptom type 1 consisted of small necrotic spots, ≤0.3 cm in diameter, with a clear, straw coloured centre and a dark edge (Figure 1a); there were also larger necrotic spots which has a rounded, elongate shape, and measured ≤2.0 cm long and 0.5 cm wide, with sharply

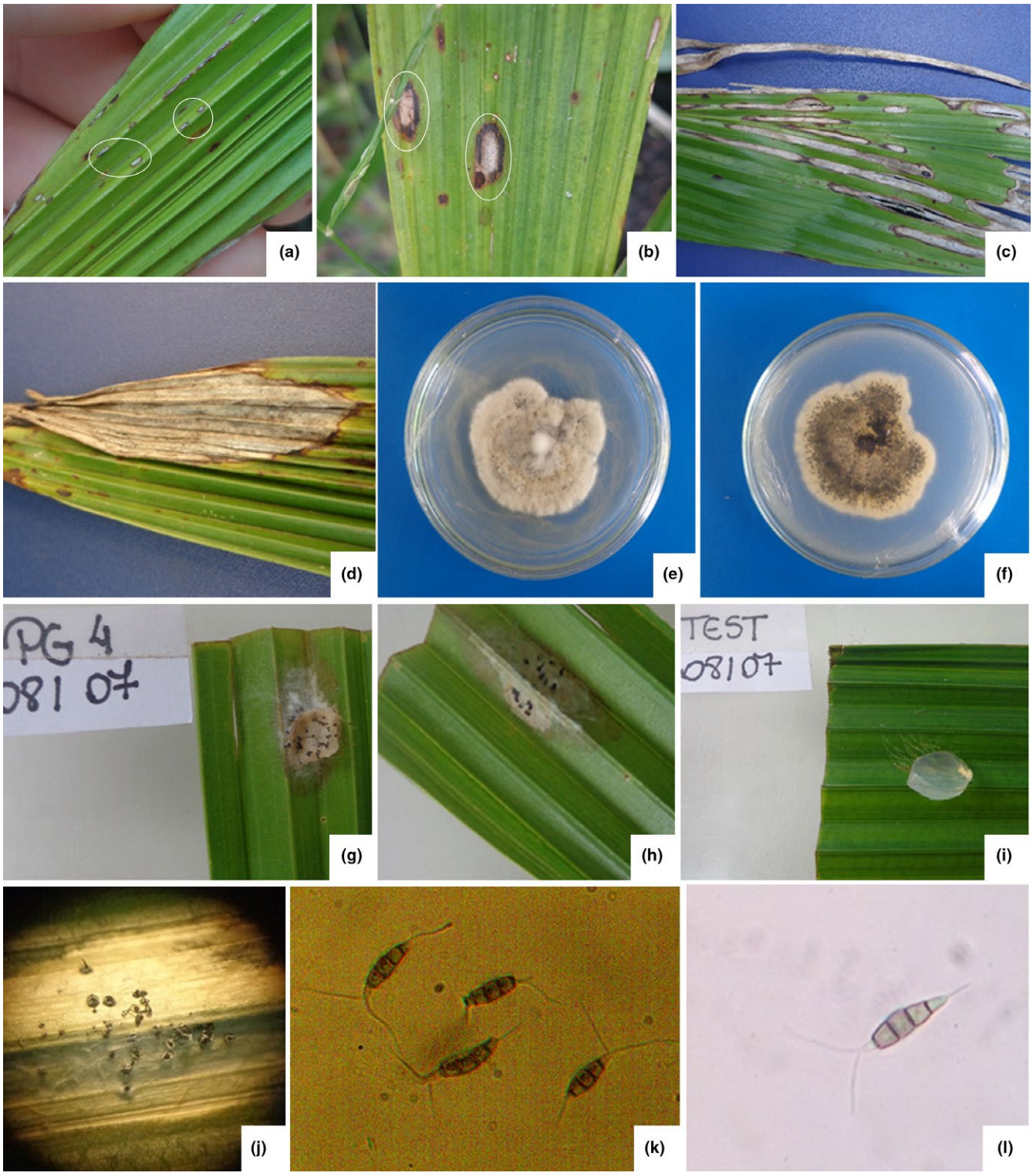

FIGURE 1 (a,b,c,d) Symptom type 1 on leaves of naturally diseased guariroba. (e,f) Mycelial growth of a *Pestalotiopsis* sp. isolated from symptom type 1 after 7 days of incubation on PDA. (g,h) Symptoms of *Pestalotiopsis* sp. on sections of detached leaves of guariroba 6 days after artificial inoculation (inoculated using pure culture on PDA plugs). (i) Control, non-inoculated leaf section. (j) Acervuli from an isolate of *Pestalotiopsis* sp. observed using a stereomicroscope (×40). (k,l) Conidia from an isolate of *Pestalotiopsis* sp. observed using an optical microscope (×100)

demarcated edges of dark colour, with small black spots noticeable in the central area of the lesion (Figure 1b). Observation under dissecting and optical microscopes revealed the black spots were acervuli containing spore masses. Coalescence of lesions on the leaf surface was also observed, resulting in large areas of necrotic leaf tissue (Figure 1c,d).

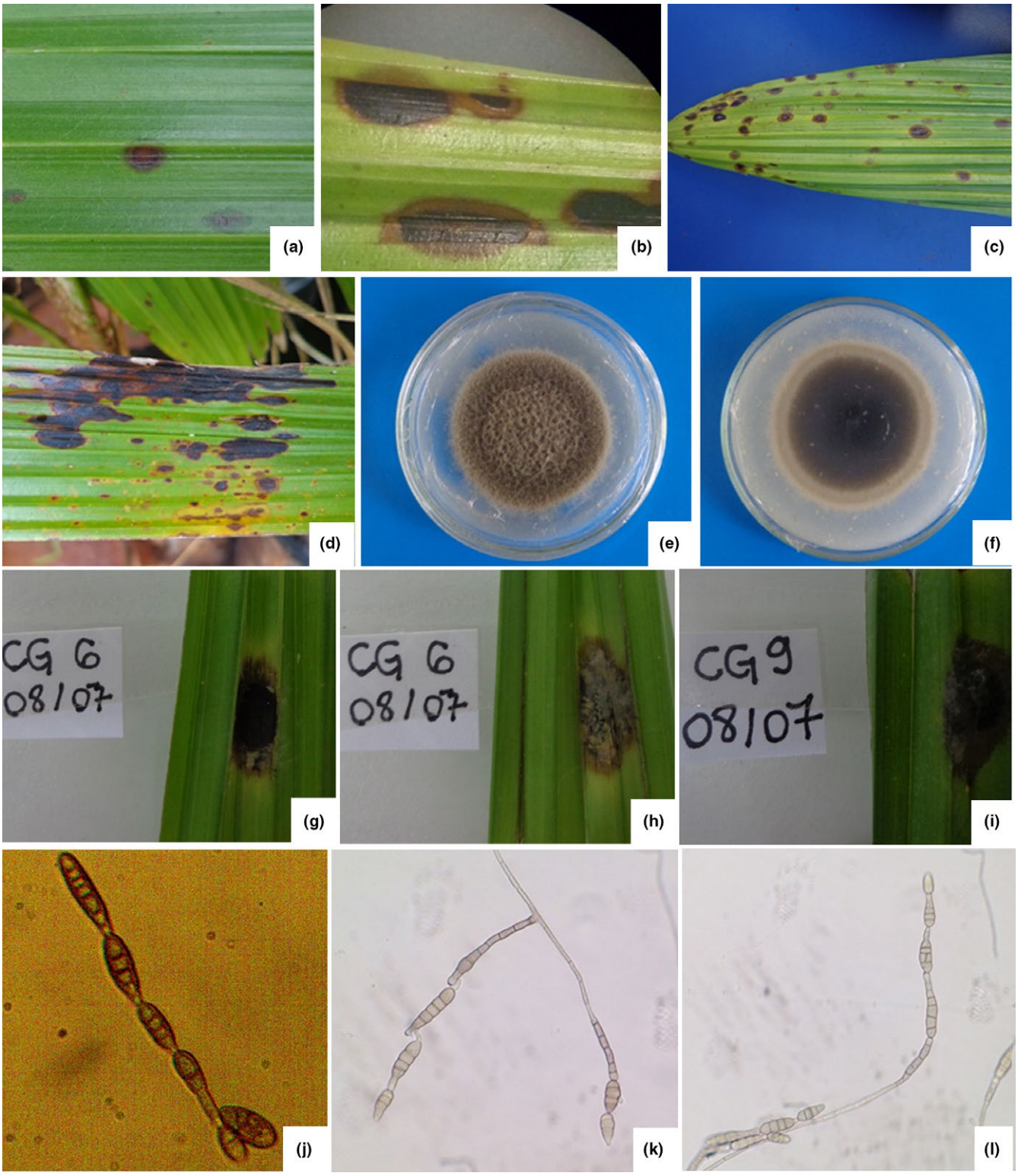

FIGURE 2 (a,b,c,d) Symptom type 2 on leaves of naturally diseased guariroba. (e,f) Mycelial growth of *Alternaria* sp. isolated from symptom type 2 after 7 days of incubation on PDA. (g,h,i) Symptoms of *Alternaria* sp. on leaf sections of guariroba 6 days after artificial inoculation (inoculated using pure culture on PDA plugs) (×40). (j,k,l) Conidiophores and conidia of *Alternaria tenuissima* isolated from guariroba seedlings and viewed using an optical microscope (×100)

Samples scraped from the leaf spots and viewed under an optical microscope revealed the presence of multiseptate hyphae of light brown colour, and large numbers of 5-celled spores; the three central cells were brown in colour and the two terminal cells hyaline, having a hair-like appendage on the apical and basal cells. Samples prepared from leaf material incubated for 24 hr in a humid chamber had the same structures as described above.

Symptom type 2 was initially manifest as dark brown pits with a rounded shape ≤0.2 cm in diameter (Figure 2a); developing to rounded irregularly shaped lesions, ≤1.2 cm long and 0.5 cm wide, with a central dark brown colour, often with a light brown halo (Figure 2b,c). Lesions could coalesce resulting in larger, diseased areas (Figure 2d).

Slides prepared from preliminary scrapes of the lesion surface and observed under an optical microscope (40×) showed multiseptate hyphae of a brown colour; and spores of oblong shape, with both longitudinal and transverse septa. Similar structures were observed on slides prepared from the surfaces of leaf material incubated for 24 hr in a humid chamber.

Some leaves showed co-occurrence of the two symptom types, indicating the pathogens could coexist on the same leaves of guariroba seedlings.

3.2 | Isolation and culture of fungi associated with leaf spots of guariroba

Four isolates from symptom type 1 were obtained in pure culture, which were maintained on PDA. All four isolates presented the same colony and morphological characteristics, with dense mycelium that was initially white in colour, but becoming grey to yellowish grey in older colonies, with the underside having a slight yellowing. Black dots formed on the colony surface in areas of older mycelial growth 4 days after inoculating the PDA, which were identified as reproductive structures resembling acervuli (Figure 1e,f).

Five isolates from symptom type 2 were grown on PDA, and all showed similar colony and morphological characteristics. After 7 days of incubation, the colonies revealed brown to olive brown in colour and had a cottony appearance (Figure 2e,f).

3.3 | Pathogenicity on leaf sections

In all cases where leaf sections were inoculated with the fungus isolated from symptom type 1, that same symptom type developed at the inoculation sites. The symptoms appeared 6 days after inoculation with the mycelial disc (Figure 1g,h). Leaf tissue in contact with the mycelium disc containing fungal structures initially became water-soaked, and later became a straw-grey colour. The control leaf sections developed no symptoms (Figure 1i). White mycelial growth developed on the surface of the necrotic area with formation of small, black, reproductive structures resembling acervuli in the centre of the necrotic area (Figure 1j).

In all cases where leaf sections were inoculated with the fungus isolated from symptom type 2, the same symptom type developed at the inoculation sites. The symptoms developed 6 days after inoculation. Dark brown to olive coloured mycelial growth was observed on the surface of the lesion (Figure 1g,h,i).

3.4 | Pathogenicity test using seedlings of guariroba

When seedlings were inoculated with a spore suspension of either fungus, symptoms developed 5 days after inoculation with both symptoms type 1 and 2, but the control treatment developed no symptoms. Symptom type 1 was characterized by small necrotic spots, up to 0.3 cm in diameter, with a clear, straw coloured centre and a dark edge, although there were also larger necrotic spots which had a rounded, elongate shape, with sharply demarcated dark-coloured edges, with small black spots noticeable in the central area of the lesion. Observation under the dissecting and optical microscopes revealed the black spots were acervuli containing spore masses.

Symptom type 2 appeared initially as dark brown coloured dots, which developed into spots approximately 0.4 cm in diameter. Thus, the process and results of isolation, morphological identification, inoculation and re-isolation procedures from leaves and from seedlings fulfilled Koch's postulate for the causal agent from both symptom types 1 and 2.

3.5 | Morphology of isolates obtained in pure culture

Fungi isolated from symptom type 1 had indistinct conidiophores, with conidia ranging from 14.2 to 26.1 (19.6 ± 2.29) μm in length and 4.8 to 5.8 (5.1 ± 0.4) μm in width. The length of the basal cell was 1.9 to 5.2 (5.1 ± 0.4) μm; the three median cells together measured between 5.1 and 16.8 (13.2 ± 1.9) μm; the length of the second basal cell was 3.4 to 6.1 (4.5 ± 0.7) μm; the third basal cell was 2.9 to 5.4 (4.2 ± 0.6) μm; the fourth basal cell was 3.4 to 5.0 (4.3 ± 0.4) μm and the apical cell was 2.2 to 5.2 (3.2 ± 0.7) μm. The number of appendices on the conidia varied between two and three (2.2 ± 0.4), and the length of the upper appendix was between 6.9 and 21.4 (14.4 ± 3.5) μm long (Figure 1k,l).

Fungi isolated from symptom type 2 had conidia that were formed in chains of up to ten conidia. The conidiophores were short, ranging from 15.6 to 49.8 (27.3 ± 8.0) μm in length and 1.8 and 4.3 (2.9 ± 0.6) μm in width. Conidia were piriform or obclavate, and 20.1 to 56.3 (35.5 ± 9.4) × 7.5 to 11.5 (9.5 ± 1.2) μm in size. The conidia contained three to five (3.5 ± 0.5) transverse septa and zero to two (0.9 ± 0.8) longitudinal septa (Figure 2j,k,l).

3.6 | Confirmation of fungal species by sequencing

PCR using the universal primers pair ITS4 and ITS5 for the ITS region resulted in amplification of a single band of approximately 600 bp for all the isolates obtained from symptom type 1 and 2.

The resulting sequence from the four isolates of symptom type 1 (Genbank accession nos. KU726830, KU726831, KU726832 and KU726833) showed 99% homology with the GenBank accessions KR056293.1 and AF409955.1, both being sequence data from *Pestalotiopsis adusta*. Thus, based on pathogenicity testing, morphological characteristics and molecular characterization we deduce that *P. adusta* is the causal agent of a *Pestalotiopsis* spot disease of *S. oleracea* in Brazil.

The resulting sequence from the five isolates of symptom type 2 (GenBank accession nos. KX130093, KX130094, KX130095, KX130096 and KX130097) showed 99% homology with accession KP942908.1 (*Alternaria tenuissima*). Thus, based on pathogenicity testing, morphological characteristics and molecular characterization

we deduce that *A. tenuissima* is the causal agent of an *Alternaria* spot disease of *S. oleracea*.

4 | DISCUSSION

Based on the fulfilment of Koch's postulate, we conclude that the two isolated, cultured fungi were the causal agents of the two symptom types observed in the field in nurseries of guariroba in the state of Minas Gerais. No previous reports were found of these two pathogens (*P. adusta* and *A. tenuissima*) on guariroba, confirming that this is the first formal report of these pathogens infecting this host.

The results of the morphological and molecular analysis indicated that *P. adusta* was the causal agent of symptom type 1 on *S. oleracea*. Diseases caused by *P. adusta* have been reported previously on foliage or fruit of other species in the Arecaceae (Frölich, Hyde, & Guest, 1997; Rosado, Machado, & Pereira, 2015; Russomanno, Kruppa, & Coutinho, 2007), and there are reports of other *Pestalotiopsis* sp. that infect other species of palm, including peach palm (*Bactris gasipaes*) and the ruffled fan leaf palm (*Licuala grandis*). In addition, oil palm (*Elaeis guineensis*) is reported to be infected by *Pestalotiopsis theae* in Thailand (Suwannarach, Sujarit, Kumla, Bussaban, & Lumyong, 2013). *Pestalotiopsis adusta* has previously been reported infecting coconut palm (*Cocos nucifera*) (Russomanno et al., 2007; Sologuren & Juliatti, 2007). Comparing the symptoms of disease caused by *P. adusta* on *S. oleracea* with disease symptoms described by Russomanno et al. (2007) indicates they bear some resemblance to each other. Characteristics of the pathogen *P. adusta* observed in the current study correspond well with the observations of Jeewon, Liew, and Hyde (2002) and Maharachchikumbura et al. (2012). Seven-day-old colonies of *Pestalotiopsis* have dense white mycelium, and form black fruiting bodies, and have a yellowish underside to the colony. Jeewon et al. (2002) and Maharachchikumbura et al. (2012) claimed that spores and associated structures are the most prominent morphological characters for identification. Their morphological characterization of *P. adjusta* is congruent with the structures we observed based on sample preparations of symptom type 1 assessed microscopically.

Furthermore, the molecular analysis based on the ITS sequence demonstrated sequence that was virtually identical to that previously characterized for *P. adjusta* (99% homology). Thus, it was concluded that the causal agent of symptom type 1 was *P. adjusta*, which resides in the fungal order Melanconiales. Although ITS sequence analysis has been used previously for phylogenetic analysis and can provide good resolution to many species of *Pestalotiopsis*, the analysis of further DNA sequences, such as the β-tubulin and TEF1 genes would provide even more definitive results (Maharachchikumbura, Guo, Chukeatirote, Bahkali, & Hyde, 2011; Maharachchikumbura et al., 2012).

Certain species of *Pestalotiopsis* may cause serious damage to ornamentals plants, and the number of known host species is increasing (Hopkins & McQuilken, 2000). Guariroba can now be included as a host. Production losses due to *Pestalotiopsis* species can be severe in some crops: for example, as much as 17% yield loss was reported in tea (*Camellia sinensis*) (Joshi, Sanjay, Baby, & Mandal, 2009) and up to 15% yield loss in cowpea (*Vigna unguiculata*) (Mahadevakumar & Janardhana, 2014).

The isolates obtained from symptom type 2 on *S. oleracea* had morphological and cultural characteristics typical for species of the genus *Alternaria*, in the order Pleosporales (Barnett & Hunter, 1998; Zheng, Zhao, Wang, & Wu, 2015). According to those authors, *Alternaria* spp. initially develop a free and cottony appearance, the colour of the colony ranging from grey–green to olive brown on PDA. Later, according to Simmons (2007) and Zheng et al. (2015), isolates of *A. tenuissima* are characterized by unbranched conidial chains with up to twelve conidia with up to one or two short lateral branches, if present, and the conidiophores and conidia presents the shape, size, number and kind of septa similar and congruent with the fungus isolated and characterized here from symptom type 2. In addition, the molecular analysis based on the ITS region sequences further confirmed that the identity of the fungus isolated from symptom type 2 was *A. tenuissima* (99% sequence homology), leading to the conclusion that the aetiology of the symptom type 2 is *A. tenuissima*. According to Woudenberg, Groenewald, Binder, and Crous (2013), the ITS sequence provides good support (95-99% based on Bayesian Posterior Probabilities and 100% by RAxML bootstrap support) to classify *Alternaria* species to the "Alternata" section, in which *A. tenuissima* belongs. Further sequence analysis, such as sequence of the translation elongation factor 1-alpha (TEF1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, can be used to provide more certainty of identity (Woudenberg et al., 2013), but based on the combined ITS sequence data, colony morphology and cultural characteristics the identity of *A. tenuissima* as the cause of symptom type 2 is compelling.

Despite many *Alternaria* species being saprophytic, some species are plant pathogens that, collectively, cause a range of diseases of great economic importance on a wide variety of host crop plants including cereals, ornamentals, oil crops and vegetables (Lawrence, Gannibal, Peever, & Pryor, 2013; Mendes & Urben, 2016; Thomma, 2003). In Brazil *A. tenuissima* is previously reported infecting lily of palm (*Cordyline cannifolia*), Taro (*Colocasia esculenta*), beans (*Vigna unguiculata*), sorghum (*Sorghum bicolor*) and eucalyptus (*Eucalyptus grandis*) (Mendes et al., 1998). This is the first report of *A. tenuissima* causing disease in guariroba.

As a causal agent of disease on many palm species and other crops, the genus *Alternaria* stands out as one of the most important that cause leaf diseases (Abdullah, Lopez Lorca, & Jansson, 2010; Ammar & El-Naggar, 2011; Maitlo, Markhand, Abul-Soad, Lodhi, & Jatoi, 2014; Sologuren & Juliatti, 2007) and fruit rots (Zaid, De Wet., Djerbi, & Oihabi., 2002). Estimates of yield losses due to *Alternaria* species were not found in palm trees, but numbers from other crops demonstrate the importance of *Alternaria* diseases: yield losses of up to 60% were reported in sunflower (Carson, 1985) and up to 58% in linseed (Singh, Singh, & Parmar, 2014).

The process of isolation, morphological identification and inoculation of guariroba to demonstrate symptom development, and subsequent re-isolation and identification of the inoculated organism fulfilled Koch's postulate, confirming that the causal agents of symptom type 1 and 2 were *P. adjusta* and *A. tenuissima*, respectively. Although occurrence of these and other diseases

is now demonstrated on guariroba, the prevalence of these diseases, its impact on production and control methods remains to be established.

Several management strategies are effective for controlling plant diseases caused by species of *Pestalotiospsis* and *Altenaria*. For *Pestalotiopsis*, systemic fungicides, such as thiophanate methyl and carbendazim, and contact fungicides, such as mancozeb and copper oxychloride, were effective for controlling the disease in tea (Sanjay, Ponmurugan, & Baby, 2008). Biocontrol agents including *Trichoderma*, *Gliocladium* and *Pseudomonas* were also efficacious (Sanjay et al., 2008). Cultural practices, including irrigation and flooring/pot disinfection also reduced disease in *Calluna vulgaris* (McQuilken and Hopkins, 2004). With *Alternaria*, control strategies include cultural practices (removing potentially infected material from the production field and ensuring seed or transplants are pathogen free) and chemical control (including Quinone outside Inhibitors and Succinate Dehydrogenase Inhibitors classes of fungicides) are highly recommended (Kemmitt, 2002). Induced resistance may also be efficacious for controlling *Alternaria* species in some plants (Morita et al., 2003; Ton, Van Pelt, Van Loon, & Pieterse, 2002).

Further research is necessary to establish the impact of these pathogens on guariroba production and the effectiveness of different methods of control, including cultural, biological and chemical approaches.

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